Celastrol, a Triterpene Extracted from the Chinese "Thunder of God Vine," Is a Potent Proteasome Inhibitor and Suppresses Human Prostate Cancer Growth in Nude Mice

Huanjie Yang,[1] Di Chen,[1] Qiuzhi Cindy Cui,[1] Xiao Yuan,[2] and Q. Ping Dou[1]

[1]The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan and [2]Wuhan Botanical Garden, Chinese Academy of Science, Wuhan, People's Republic of China

Abstract

Interest in the use of traditional medicines for cancer prevention and treatment is increasing. *In vitro, in vivo*, and clinical studies suggest the potential use of proteasome inhibitors as novel anticancer drugs. Celastrol, an active compound extracted from the root bark of the Chinese medicine "Thunder of God Vine" (*Tripterygium wilfordii* Hook F.), was used for years as a natural remedy for inflammatory conditions. Although Celastrol has been shown to induce leukemia cell apoptosis, the molecular target involved has not been identified. Furthermore, whether Celastrol has antitumor activity *in vivo* has never been conclusively shown. Here, we report, for the first time, that Celastrol potently and preferentially inhibits the chymotrypsin-like activity of a purified 20S proteasome (IC_{50} = 2.5 μmol/L) and human prostate cancer cellular 26S proteasome (at 1-5 μmol/L). Inhibition of the proteasome activity by Celastrol in PC-3 (androgen receptor- or AR-negative) or LNCaP (AR-positive) cells results in the accumulation of ubiquitinated proteins and three natural proteasome substrates (IκB-α, Bax, and p27), accompanied by suppression of AR protein expression (in LNCaP cells) and induction of apoptosis. Treatment of PC-3 tumor–bearing nude mice with Celastrol (1-3 mg/kg/d, i.p., 1-31 days) resulted in significant inhibition (65-93%) of the tumor growth. Multiple assays using the animal tumor tissue samples from both early and end time points showed *in vivo* inhibition of the proteasomal activity and induction of apoptosis after Celastrol treatment. Our results show that Celastrol is a natural proteasome inhibitor that has a great potential for cancer prevention and treatment. (Cancer Res 2006; 66(9): 4758-65)

Introduction

There is a growing public interest in the use of complementary and alternative medicine for cancer prevention and treatment (1, 2). Traditional Chinese medicine, an important component of complementary and alternative medicine, may serve as a useful model for scientific inquiry because it has a standardized system of diagnostics and therapies, and is practiced worldwide (3). *Tripterygium wilfordii* Hook F., an ivy-like vine also known as "Thunder of God Vine," belongs to the Celastraceae family and has been used as a natural medicine in China for hundreds of years (4). One of the active components extracted from *T. wilfordii* Hook F. is Celastrol, a quinone methide triterpene (Fig. 1*A*). Due to its antioxidant or antiinflammatory effects, Celastrol has been effectively used in the treatment of autoimmune diseases, asthma, chronic inflammation, and neurodegenerative disease (5–12). Under *in vitro* conditions, Celastrol was found to inhibit cancer cell proliferation and induce leukemic cell death (13–15); however, the molecular mechanism involved still remains unclear. Furthermore, whether Celastrol has antitumor activity *in vivo* has never been conclusively shown.

Protein degradation, which is as essential to the cell as protein synthesis, occurs via two pathways: lysosome-mediated and ubiquitin/proteasome-mediated pathways (16, 17). The 26S proteasome is made of two 19S regulatory particles and a 20S core particle. The latter is a multicatalytic threonine protease with at least three distinct catalytic activities, chymotrypsin-like (cleavage after hydrophobic residues and mediated by β5 subunit), trypsin-like (cleavage after basic residues by β2 subunit), and peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like (cleavage after acidic residues by β1 subunit) activities. By acting as a tag, a chain of ubiquitin can target a substrate protein to the 26S proteasome for destruction (16, 17). The ubiquitin/proteasome-dependent pathway is known to degrade many endogenous proteins including transcription factors, cyclins, tumor suppressor proteins, and misfolded or damaged proteins (18–21). Several studies have shown that inhibition of the proteasomal chymotrypsin–like activity resulted in the accumulation of several target proteins (i.e., IκB-α, Bax, and p27) and induction of apoptosis in various types of tumor cells (22–25).

Previously, we reported that some dietary flavonoids (i.e., Quercetin; Fig. 1*A*) containing an aromatic ketone structure were able to inhibit the proteasomal chymotrypsin-like activity (26). We proposed that the aromatic ketone carbon would interact with the hydroxyl group of the *N*-threonine of the proteasomal β5 subunit, forming a covalent bond and causing inhibition of the proteasomal chymotrypsin-like activity (26). We noticed that Celastrol contains a conjugated ketone in its A-ring that is similar to the one in Quercetin's C-ring (Fig. 1*A*). We therefore hypothesized that Celastrol might be a proteasome inhibitor.

In the present study, we report, for the first time, that Celastrol directly inhibits the chymotrypsin-like activity of a purified rabbit 20S proteasome (IC_{50} = 2.5 μmol/L) and 26S proteasome in intact androgen-independent PC-3 (androgen receptor- or AR-negative) and androgen-dependent LNCaP (AR-positive) prostate cancer cells (at 1-5 μmol/L). Inhibition of the proteasome by Celastrol in PC-3 or LNCaP cells results in the accumulation of ubiquitinated proteins and natural proteasome substrates, IκB-α, Bax, and p27, and/or suppression of AR protein expression. Following proteasome inhibition, apoptotic cell death was induced. Furthermore,

Requests for reprints: Q. Ping Dou, The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, 640.1 HWCRC, 4100 John R Road, Detroit, Michigan, 48201. Phone: 313-576-8301; Fax: 313-576-8307; E-mail: doup@karmanos.org.

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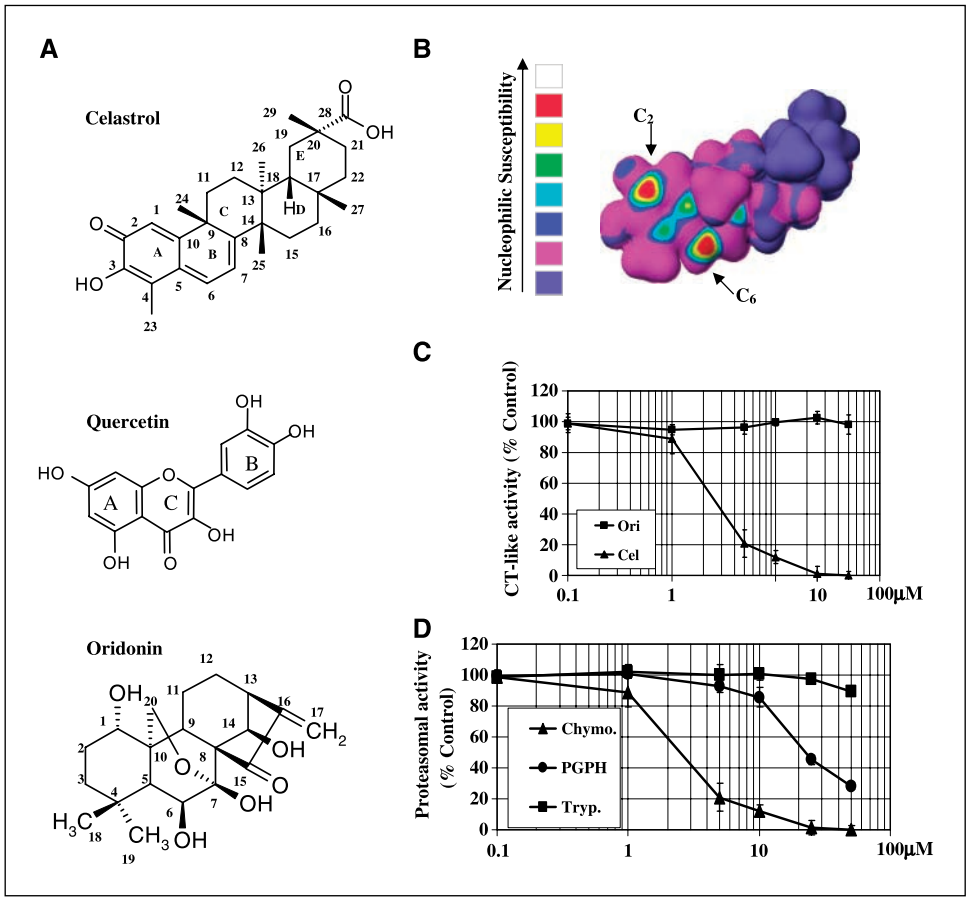

Figure 1. Inhibition of the chymotrypsin-like activity of a purified rabbit 20S proteasome activity by Celastrol. *A,* the chemical structures of Celastrol, Quercetin, and Oridonin. *B,* nucleophilic susceptibility of Celastrol analyzed using CAChe software. Higher susceptibility was shown at the C_2 and C_6 positions of Celastrol. *C* and *D,* potency and selectivity of Celastrol. To determine whether Celastrol could inhibit the proteasome activity directly, a purified 20S rabbit proteasome (0.1 μg) was incubated with various peptide substrates for the proteasomal chymotrypsin-like, PGPH-like, and trypsin-like (*Tryp*) activities in the presence of Celastrol or Oridonin at the indicated concentrations, as described in Materials and Methods.

treatment of prostate tumor–bearing nude mice with Celastrol (1-3 mg/kg/d, i.p., for 1-31 days) resulted in significant tumor growth inhibition and massive apoptosis induction, associated with *in vivo* proteasome inhibition and AR suppression. Our results show that Celastrol is a natural proteasome inhibitor that has great potential for the prevention and treatment of human cancers.

Materials and Methods

Materials. Purified Celastrol (>98%) and Oridonin (>98%) were a generous gift from π-π Technologies, Inc. (Shenzhen, China) and were used for a portion of the *in vitro* and *in vivo* experiments. Additional purified Celastrol (>98%) and Oridonin (>98%) were purchased from Calbiochem, Inc., (San Diego, CA) for further experiments until the completion of the report. Both Celastrol and Oridonin were dissolved in DMSO (Sigma, St. Louis, MO) at a stock concentration of 50 mmol/L, aliquoted, and stored at −20°C. Purified rabbit 20S proteasome, fluorogenic peptide substrates Suc-LLVY-AMC, Z-LLE-AMC, Z-ARR-AMC (for the proteasomal chymotrypsin-like, PGPH-like, trypsin-like activities, respectively) and Ac-DEVD-AMC (for caspase-3/-7) were from Calbiochem. Another fluorogenic peptide substrate Z-GGL-AMC (specific for the proteasomal chymotrypsin-like activity) and mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP) were from BIOMOL International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), ubiquitin (P4D1), AR (441), rabbit polyclonal antibody against inhibitor of nuclear factor κB-α (IκB-α; C-15), and goat polyclonal antibody against actin (C-11) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody NCL-p27 was purchased from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom). Apoptag Peroxidase *In situ* Apoptosis Detection Kit was from Chemicon International, Inc. (Temecula, CA). Fetal bovine serum was from Tissue Culture Biologicals (Tulare, CA). RPMI 1640, penicillin, and streptomycin were from Invitrogen Co. (Carlsbad, CA), enhanced chemiluminescence reagent was from Amersham Biosciences (Piscataway, NJ), and Cremophor EL and other chemicals were from Sigma.

Cell culture and whole cell extract preparation. PC-3 and LNCaP cells, purchased from American Type Culture Collection (Manassas, VA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin and maintained at 37°C and 5% CO_2. A whole cell extract was prepared as described previously (22).

Nucleophilic susceptibility analysis. The electron density surface colored by nucleophilic susceptibility was created with the use of Quantum CAChe (Fujitsu, Fairfield, NJ) by performing a nuclear susceptibility analysis using the PM5 geometry and PM5 wave function in water as described previously (24). A colored "bull's-eye" with a red center denotes atoms that are highly susceptible for nucleophilic attack.

Inhibition of purified 20S proteasome activity by Celastrol. A purified rabbit 20S proteasome (0.1 μg) was incubated with 40 μmol/L of various fluorogenic peptide substrates in 100 μL assay buffer [20 mmol/L Tris-HCl (pH 7.5)], in the presence of Celastrol or Oridonin at different concentrations or in the solvent DMSO for 2 hours at 37°C, followed by measurement of inhibition of each proteasomal activity (24–26).

Inhibition of the proteasome activity in intact prostate tumor cells by Celastrol. Prostate cancer cells (5,000-8,000) were plated in each well of a 96-well plate and then treated with either DMSO, Celastrol, or Oridonin at different concentrations for 12 to 16 hours, followed by an additional 2-hour incubation with Z-Gly-Gly-Leu-AMC (at 40 μmol/L). After that, the proteasome activity was measured using the whole plate as described above.

Caspase-3 (or caspase-7) activity assay and Western blot analysis. Prostate cancer cells were treated with Celastrol or Oridonin as indicated in the legends. The prepared whole cell extracts (30 μg per sample) were then

incubated with 40 μmol/L of Ac-DEVD-AMC in 100 μL assay buffer at 37°C for at least 2 hours. The release of the AMC groups was measured as described above. Western blot assay using enhanced chemiluminescence reagent was done as previously described (26).

Human prostate tumor xenograft experiments. Male nude immunodeficient mice NCRNU-M, aged 5 weeks, were purchased from Taconic Research Animal Services (Hudson, NY) and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. On day 0, human prostate cancer PC-3 or C4-2B cells ($5\text{-}10 \times 10^6$) suspended in 0.1 mL of serum-free RPMI 1640 were inoculated s.c. in the right flank of each mouse (four mice per group). For the first experiment using PC-3 cells, on day 14 after inoculation, the animals started daily i.p. injection with either 50 to 100 μL of a vehicle [10% DMSO, 70% Cremophor/ethanol (3:1), and 20% PBS], and 1.0 or 3.0 mg/kg of Celastrol. Tumor sizes were measured daily using calipers and their volumes were calculated using a standard formula: $\text{width}^2 \times \text{length} / 2$. Body weight was measured weekly. To study whether the proteasome is inhibited in an early phase of the experiment, after 3 days of treatment, one control and one 3.0 mg/kg Celastrol–treated mouse was sacrificed. The rest were sacrificed after 16 days of treatment when control tumors reached ~1,400 mm^3. For the second PC-3 tumor experiment, 12 days after inoculation, mice were randomly divided into three groups and treated with either control, Celastrol, or Oridonin at 1.5 mg/kg daily for the duration of the study (31 days). In another experiment, to study the effects of Celastrol on AR expression, nude mice bearing C4-2B tumors received daily i.p. injection of the vehicle or 3.0 mg/kg Celastrol.

Terminal nucleotidyl transferase–mediated nick end labeling, immunostaining, and other assays using tumor tissue samples. Terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay using *in situ* apoptosis detection kit and immunostaining of p27 were done as described previously (27). The proteasomal or caspase activity assays and Western blotting using animal tumor samples were done similarly as described above using cultured prostate cancer cells.

Statistical analysis. Student's *t* test was applied to evaluate the differences between treated and control animal groups with respect to tumor growth. Data from multiple groups were analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparison. For all the tests, the level of significance was set at $P < 0.05$.

Results

Inhibition of the chymotrypsin-like activity of a purified rabbit 20S proteasome by Celastrol but not Oridonin. Most recently, we reported that some flavonoids, such as Quercetin and Kaempferol, have proteasome-inhibitory activities and that their aromatic ketone structure may play a direct role in this inhibition (ref. 26; Fig. 1*A*). We noticed that the A-ring structure of Celastrol, containing a ketone conjugated with four double bonds, is very similar to that of the C-ring of Quercetin (Fig. 1*A*). We therefore hypothesize that Celastrol is a proteasome inhibitor. To test this hypothesis, we first did computational electron density analysis for the Celastrol molecule. When the atomic orbital energy was analyzed, C_2 on A-ring and C_6 on B-ring of Celastrol showed a high susceptibility toward a nucleophilic attack (Fig. 1*B*), suggesting that one or both of these carbons could interact with and inhibit the proteasome (24, 26). Oridonin is a diterpenoid that is extracted from the Chinese herb *Rabdosia rubescens* (28). We noted that Oridonin also contains a ketone that is conjugated with only one double bond (C_{15}-C_{17}, Fig. 1*A*). We therefore speculated that Oridonin might be a weak proteasome inhibitor. Consistently, relatively low nucleophilic susceptibility was found in Oridonin (data not shown).

To provide direct evidence for proteasome inhibition by Celastrol, we did a cell-free proteasome activity assay using a purified rabbit 20S proteasome in the presence of Celastrol or Oridonin at various concentrations. The chymotrypsin-like activity of the purified 20S proteasome was significantly inhibited by Celastrol with an IC_{50} value of 2.5 μmol/L (Fig. 1*C*). In contrast, Oridonin at up to 50 μmol/L only had a slight inhibitory activity in the purified proteasome (Fig. 1*C*).

To investigate whether Celastrol specifically inhibits the proteasomal chymotrypsin-like activity, its effects on the PGPH-like and trypsin-like activities (16, 17) of the purified 20S proteasome were examined. Celastrol at 5 μmol/L inhibited the chymotrypsin-like, PGPH-like, and trypsin-like activities of the purified 20S proteasome by 80%, 5%, and <1%, respectively (Fig. 1*D*); whereas at 10 μmol/L, it inhibited these three proteasomal activities by ~90%, 15%, and <1%, respectively (Fig. 1*D*). Therefore, it seems that Celastrol preferentially inhibits the proteasomal chymotrypsin-like activities *over* other activities.

Inhibition of cellular proteasomal activity by Celastrol is associated with apoptosis in androgen-independent PC-3 prostate cancer cells. To investigate whether Celastrol inhibits tumor cellular proteasome activity, androgen-independent, AR-negative PC-3 prostate cancer cells were treated with various concentrations (0.5-5 μmol/L) of Celastrol or Oridonin (as a comparison), followed by measuring proteasome inhibition by the cellular proteasomal chymotrypsin-like activity assay and accumulation of ubiquitinated proteins and three proteasome target proteins (IκBα, Bax, and p27). We found that Celastrol significantly inhibited the proteasomal chymotrypsin activity in PC-3 cells in a concentration-dependent manner (Fig. 2*A*); at 2.5 μmol/L it reached ~55% inhibition, comparable to its potency to a purified 20S proteasome (IC_{50} = 2.5 μmol/L; Fig. 1*C*). Under the same conditions, Oridonin at the tested concentrations showed only slight inhibitory effect (10-20%; Fig. 2*A*). Consistently, levels of ubiquitinated proteins were accumulated in PC-3 cells treated with Celastrol in a concentration-dependent manner, but were increased only slightly in those treated with Oridonin (Fig. 2*B*). Furthermore, we observed increased levels of IκB-α, Bax, and p27, three well known target proteins of the proteasome (16, 17) in PC-3 cells treated with Celastrol, but not Oridonin (data not shown, but see Fig. 3*B*). These results support the conclusion that Celastrol inhibits the cellular proteasome activity in intact PC-3 cells.

It has been shown that inhibition of tumor cellular proteasome activity is associated with apoptosis induction (22, 23). In the aliquots of PC-3 cells treated with either Celastrol or Oridonin, we measured apoptotic cell death by caspase-3 activity levels, PARP cleavage, and cellular apoptotic morphologic changes (condensation and fragmentation). Celastrol at 2.5 to 5 μmol/L induced caspase-3 activity by 4.7-fold to 5.5-fold (Fig. 2*C*). Although low levels of p85/PARP fragments were detected when 2.5 μmol/L of Celastrol was applied, almost all the PARP protein was cleaved in cells treated with 5 μmol/L of Celastrol (Fig. 2*B*). Consistently, cell death was observed when 2.5 to 5 μmol/L of Celastrol was used (Fig. 2*D*). In sharp contrast, Oridonin, at even the highest concentration used (5 μmol/L), failed to induce caspase activation, PARP cleavage, and cell death (Fig. 2*B-D*).

If inhibition of the proteasome by Celastrol is responsible for tumor cell apoptosis, we should observe inhibition of proteasomal activity prior to cell death. To test this idea, PC-3 cells were treated with 5 μmol/L of Celastrol or Oridonin for 1 to 12 hours, followed by the measurement of proteasome inhibition and apoptosis (Fig. 3). We found that the levels of ubiquitinated proteins accumulated as early as 1 hour after the addition of Celastrol, and reached their peak at 4 hours (Fig. 3*A*). Also, in Celastrol-treated

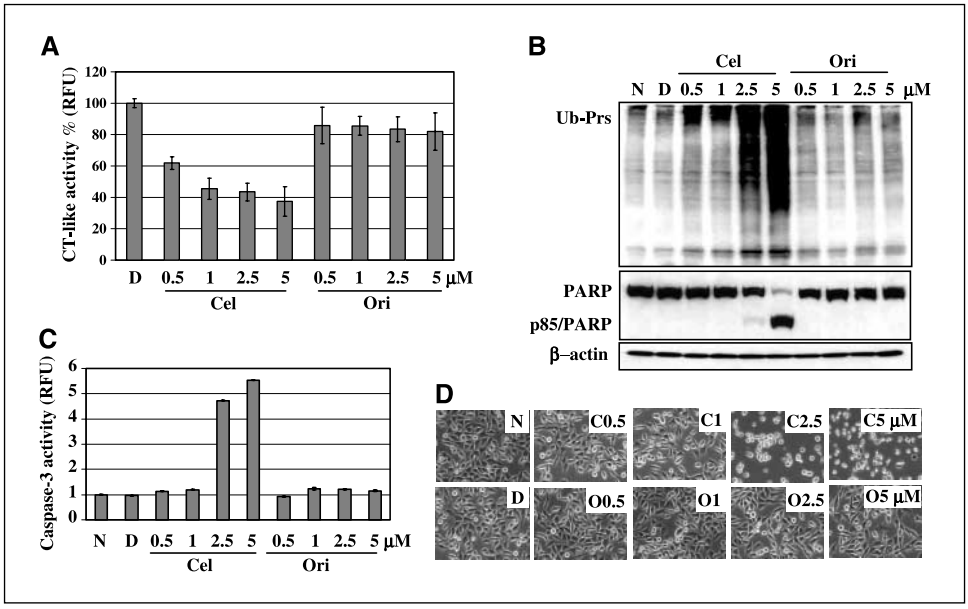

Figure 2. Dosage-effects of Celastrol on PC-3 cells. PC-3 cells (nontreated or *N*) were treated with either solvent DMSO (*D*) or different concentrations of Celastrol (*Cel* or *C*) or Oridonin (*Ori* or *O*) for 4 hours (for ubiquitinated proteins) or 12 hours, followed by measuring inhibition of the proteasomal chymotrypsin-like activity using Z-GGL-AMC (*A*), accumulation of ubiquitinated proteins (*Ub-Prs*) and cleavage of PARP (*B*), caspase-3 activation (*C*), and apoptotic morphologic changes (*D*). Molecular weight of intact PARP (116 kDa) and the cleaved PARP fragment (85 kDa). *A* and *C, columns,* mean of independent triplicate experiments; *bars,* SD.

PC-3 cells, the levels of the proteasome target proteins, IκB-α and Bax, were increased after 1 hour and further increased to its peak for 4 to 12 hours, whereas that of another proteasome target, p27, increased after 4 hours and further increased for 8 to 12 hours (Fig. 3*B*). When the PC-3 cells treated with Oridonin were examined in the same assays, much fewer effects were observed, although the expression of IκB-α and Bax was indeed increased (Fig. 3*B*), consistent with the prediction that Oridonin is a weak proteasome inhibitor (Fig. 1*A*).

In the same kinetic experiment using Celastrol, apoptosis was detected at 4, 8, and 12 hours since increased levels of caspase-3 activity (up to 5.5-fold; Fig. 3*C*) and PARP cleavage (Fig. 3*B*) were observed at these time points. Morphologically, the Celastrol-treated cells congregated as early as 1 hour and became fragmented after 4 hours (see Fig. 2*D*; data not shown). Again, during the 12-hour treatment, Oridonin at 5 μmol/L only slightly induced caspase activation and caused few apoptotic morphologic changes (Fig. 3*B* and *C*; data not shown).

Inhibition of the proteasomal activity, suppression of AR protein expression, and induction of apoptosis by Celastrol, but not Oridonin, in androgen-dependent LNCaP prostate cancer cells. AR plays an essential role for prostate cancer cell proliferation and survival (29, 30). It has been shown that proteasome inhibition was able to reduce the levels of AR protein expression (31). If Celastrol is a natural proteasome inhibitor, we would expect that it should cause inhibition of AR protein expression. To test this hypothesis, androgen-dependent, AR-positive prostate cancer LNCaP cells were treated with Celastrol or Oridonin at various concentrations (Fig. 4*A* and *B*). Again, Celastrol treatment induced proteasome inhibition, as shown by the decreased levels of chymotrypsin-like activity (by 40% at 2.5 μmol/L; Fig. 4*A*) and increased accumulation of ubiquitinated proteins (data not shown, but see Fig. 4*C*). Associated with this, AR protein expression was decreased by 2.5 μmol/L of Celastrol and almost completely inhibited by 5 μmol/L of Celastrol (Fig. 4*A*). Proteasome inhibition and AR suppression were accompanied by apoptosis induction in the Celastrol-treated LNCaP cells, as shown by increased levels of caspase-3 activity (up to 3.5-fold), PARP cleavage, and apoptotic morphology (Fig. 4*A* and *B*). Compared with Celastrol, Oridonin had much fewer proteasome-inhibitory effects and was unable to either decrease AR expression or induce apoptotic cell death under the experimental conditions tested (Fig. 4*A* and *B*).

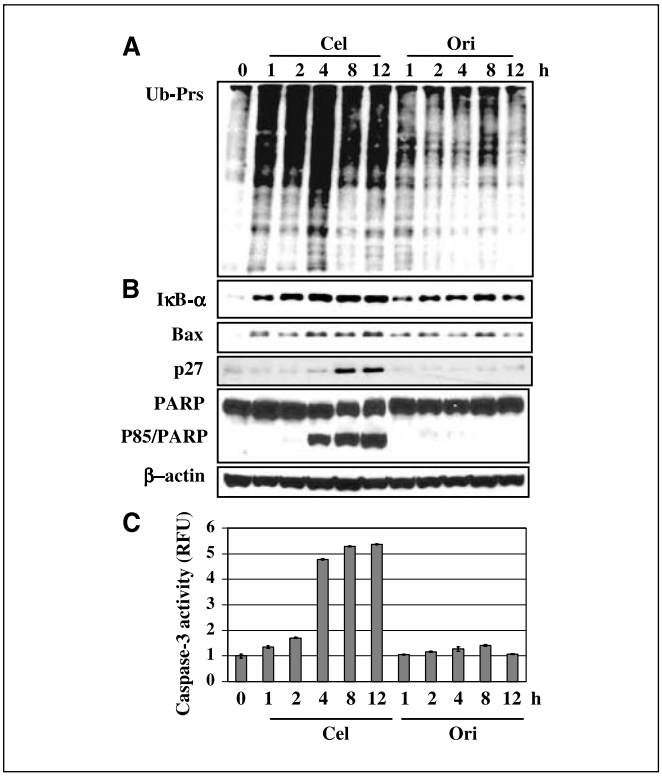

Figure 3. Kinetic studies on proteasome inhibition and apoptosis induction by Celastrol in PC-3 cells. Exponentially grown PC-3 cells (0 hours) were exposed to 5 μmol/L Celastrol (*Cel*) or Oridonin (*Ori*) for the indicated times, followed by Western blot assay using specific antibodies to ubiquitin (*A*), IκB-α, Bax, p27, PARP, and β-actin (*B*). Molecular weight of IκB-α, Bax, and p27 are 37, 23 and 27 kDa, respectively. β-Actin was used as a loading control. Fluorescent detection of caspase-3 activity (*C*) was measured using an activity assay (see Materials and Methods). *Columns,* mean of representative independent triplicate experiments; *bars,* SD.

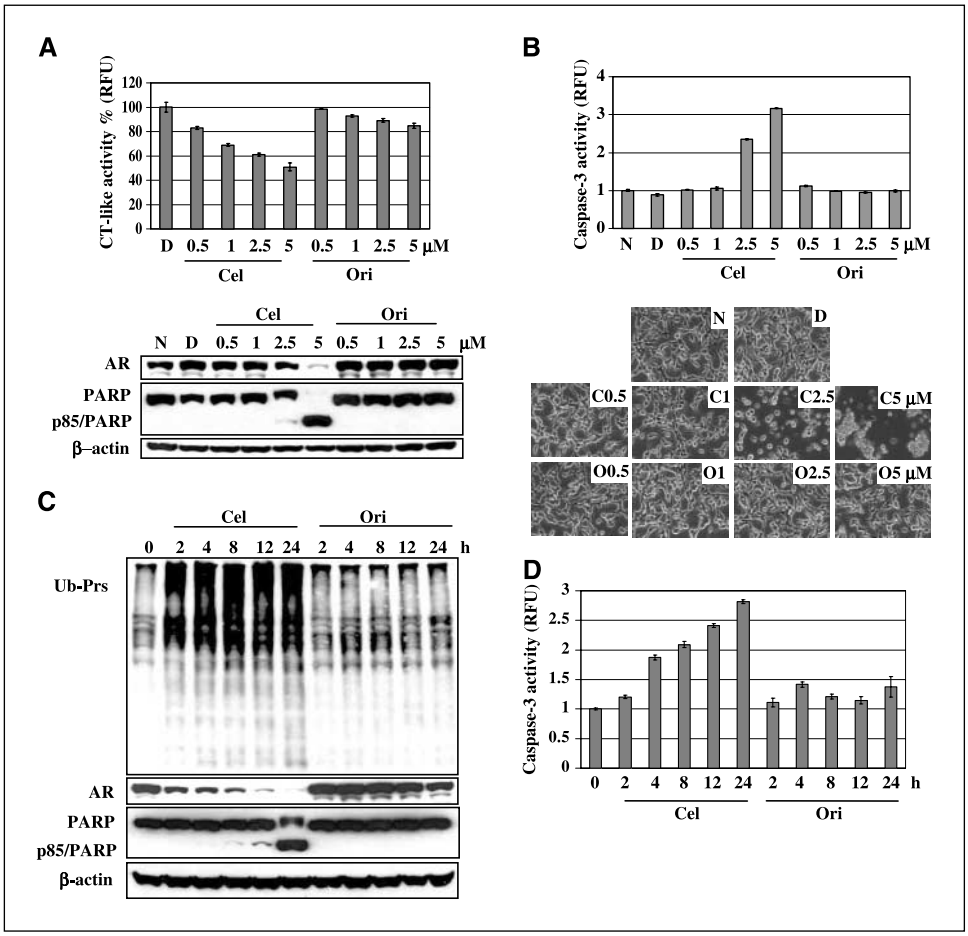

Figure 4. Dosage and kinetic effects of Celastrol on LNCaP cells. *A* and *B,* LNCaP cells (nontreated or *N*) were treated with either DMSO (*D*) or Celastrol (*Cel* or *C*) or Oridonin (*Ori* or *O*) at the indicated concentrations for 16 hours, followed by the proteosomal chymotrypsin-like activity assay using Z-GGL-AMC, or for 24 hours, followed by cell-free caspase-3 activity assay and Western blot analysis. Inhibition of chymotrypsin-like activity, loss of AR expression and cleavage of PARP (*A*), activation of caspase-3 and apoptotic morphologic changes (*B*). Molecular weight of AR is 110 kDa. *Columns,* mean of independent triplicate experiments; bars, SD. *C* and *D,* exponentially grown LNCaP cells (0 hours) were treated with 5 μmol/L Celastrol (*Cel*) or Oridonin (*Ori*) for the indicated times, followed by Western blot analysis using specific antibodies to ubiquitin, AR, PARP, and β-actin (*C*), and cell-free caspase-3 activity assay (*D*). *Columns,* mean of independent triplicate experiments; *bars,* SD.

In a kinetic experiment using LNCaP cells, both accumulation of ubiquitinated proteins and decrease in AR protein expression occurred as early as 2 hours after Celastrol treatment (Fig. 4*C*). AR levels were further decreased after 4 hours and became almost completely lost after 12 and 24 hours (Fig. 4*C*). This was followed by increased caspase activation and PARP cleavage (Fig. 4*C* and *D*). Oridonin, under the same conditions, had much fewer effects on the accumulation of ubiquitinated proteins, inhibition of AR protein expression, and induction of apoptotic cell death (Fig. 4*C* and *D*). Therefore, Celastrol is able to inhibit the proteasome activity and suppress AR protein expression, leading to apoptosis induction in LNCaP prostate cancer cells.

LNCaP cells are androgen-dependent and the cellular AR expression can be increased by androgen treatment (29, 30). To investigate whether androgen can block Celastrol-mediated AR reduction and apoptosis, LNCaP cells were pretreated with dihydrotestosterone at 10 nmol/L for 2 hours, followed by cotreatment with Celastrol at 5 μmol/L for an additional 24 hours. We found that dihydrotestosterone treatment partially inhibited both Celastrol-induced AR reduction and apoptosis (data not shown), supporting the requirement of AR in prostate cancer cell survival (29, 30).

Celastrol treatment significantly inhibits the growth of prostate cancer xenografts associated with inhibition of the proteasomal activity and induction of apoptosis *in vivo*. The data described above clearly shows that Celastrol is a natural proteasome inhibitor and apoptosis inducer in cultured prostate cancer cells. To determine whether Celastrol could inhibit proteasome activity, induce apoptosis, and inhibit tumor growth *in vivo*, we implanted PC-3 cells s.c. in nude mice. When the tumors became palpable (~ 200 mm^3), the mice were treated i.p. with either vehicle control or Celastrol at 1.0 or 3.0 mg/kg/d. Significant inhibition (up to 70%) of tumor growth by Celastrol was observed after 1 to 3 days (Fig. 5*A*), indicating that Celastrol has potent antitumor activity (see below).

To determine whether Celastrol hits its target, the proteasome, in an early phase of treatment, after 3 days of treatment, one control and one 3.0 mg/kg Celastrol–treated mouse was euthanized, and the tumors were removed and used for the preparation of tissue extracts. Figure 5*B* shows that the Celastrol-treated tumor contained only 25% of the proteasomal chymotrypsin activity as observed in the control tumor. Consistently, increased p27 levels were observed in the tissue extract of Celastrol-treated versus control tumors (Fig. 5*B*). A very strong band of ~p70 was also detected by the same anti-p27 antibody in the tumor extract of Celastrol-treated, but not the control, mouse (Fig. 5*B*). This p70 may contain ubiquitinated p27 because a similar ubiquitinated form of p27 was reported (18, 22). Thus, i.p. injected Celastrol was able to reach and inhibit the intended target, the proteasome, in a short period of time in PC-3 tumors *in vivo*. Furthermore, a PARP cleavage fragment was found in the tumor extract of Celastrol-treated mouse, but not the control (Fig. 5*B*), suggesting activation of cell death program in PC-3 tumors by Celastrol treatment.

After treatment for 16 days, control tumors grew to an average size of 1,400 ± 110 mm^3. In contrast, tumors from 1.0 and 3.0 mg/kg/d Celastrol–treated animals grew to an average size of 510 ± 158 and 250 ± 18 mm^3, corresponding to 65% and 82% inhibition, respectively ($P < 0.01$; Fig. 5*A*). The collected PC-3 tumors were then used for proteasome activity and apoptosis assays. The tumor proteasome activity was greatly inhibited at the end of the experiment because (*a*) the decreased chymotrypsin-like activity was detected (45% and 30% of the controls, respectively) in tumors treated by Celastrol at 1.0 and 3.0 mg/kg/d; (*b*) increased levels of putative ubiquitinated forms of p27 (data not shown) and Bax (Fig. 5*C*; ref. 32) were found in Celastrol-treated, but not control tumors; (*c*) immunostaining confirmed the increased expression of p27 in tumors treated with Celastrol at 3.0 mg/kg/d (Fig. 6*A*). TUNEL analysis showed that Celastrol-treated tumor cells were apoptotic (Fig. 6*B*). Consistently, the appearance of various PARP cleavage fragments was found in the Celastrol-treated, but not control, tumors (data not shown).

To determine whether a greater antitumor activity of Celastrol could be detected if it is introduced in an earlier phase of tumor development, in the second experiment, when the PC-3 tumors reached a size of ~100 mm^3, the mice were i.p. treated daily with either vehicle control, Celastrol (1.5 mg/kg), or Oridonin (1.5 mg/kg; as a comparison). We observed that after 5 days, one Celastrol-treated tumor became undetectable, and this mouse remained tumor-free to the end of the experiment, as confirmed by anatomic evidence and H&E staining (data not shown; Fig. 5*D*). After 1 month of daily treatment, control tumors grew to an average size of 1,400 ± 60 mm^3, whereas Celastrol-treated tumors grew only to an average size of only 100 ± 42 mm^3 (Fig. 5*D*), demonstrating a statistically significant ($P < 0.01$) tumor growth inhibition (93%). Oridonin, at the concentration used, had no inhibitory effect (data not shown). In addition, tumor extracts from the Celastrol-treated mice contained only 30% of the proteasomal chymotrypsin-like activity of the control- or Oridonin-treated animals, and apoptosis-specific TUNEL positivity was found only in PC-3 tumors treated with Celastrol, but not vehicle or Oridonin (Fig. 6*C*).

To study the effects of Celastrol on AR expression *in vivo*, C4-2B tumors were induced in nude mice. Injection of Celastrol (at 3.0 mg/kg/d) for 3 days caused 35% of tumor inhibition. Associated with decreased proteasome activity, we also detected decreased expression of AR protein, along with a dramatic reduction of a p50 fragment detectable by the same AR antibody, in C4-2B tumors treated with Celastrol, compared with the control (Fig. 5*C*). Finally, increased Caspase-3 activity (data not shown) and TUNEL positivity (Fig. 6*D*) were observed only in the Celastrol-treated C4-2B tumors. Taken together, these data show that Celastrol is able to inhibit the proteasomal activity, suppress AR expression, and induce apoptosis in prostate tumors *in vivo*, which may be responsible for the antitumor activity observed.

Discussion

The search for new antitumor drugs from natural sources is one of the most important approaches for cancer prevention and therapy. Whether the medicinal component Celastrol has antitumor activity, and what its target is, have not been studied previously (13–15). In the current study, we have shown that Celastrol is a potent inhibitor of the proteasomal chymotrypsin-like activity both *in vitro* (Fig. 1), in cultured prostate tumor cells (Figs. 2-4), and in animals (Figs. 5 and 6). Consistent with previous reports (22, 23), the current study has also shown that inhibition of the proteasomal chymotrypsin-like activity by Celastrol in prostate cancer cells and xenografts is associated with its apoptosis-inducing and/or antitumor activities (Figs. 2-6).

It has been shown that the ester bond carbon of β-lactone is responsible for potently and specifically inhibiting the proteasome

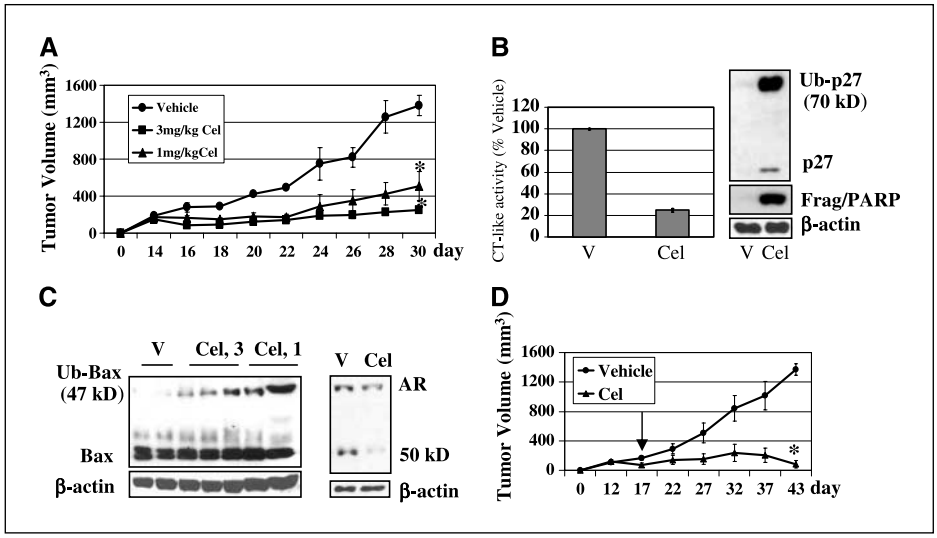

Figure 5. Proteasome-inhibitory and antitumor effects of Celastrol *in vivo*. *A* to *C,* male nude mice bearing PC-3 tumors were treated on day 14 with either control vehicle (*V*) or Celastrol (*Cel*) at 1.0 or 3.0 mg/kg/d to day 30 (the first PC-3 experiment). *A,* PC-3 tumor inhibition by Celastrol. *Points,* mean tumor volume in each experimental group containing four mice; *bars,* SE; *, $P < 0.01$. *B,* early effects of Celastrol. After 3 days of treatment, tumors were removed from one control and one 3 mg/kg Celastrol–treated mouse and the prepared tissue extracts were used for the proteasomal chymotrypsin-like activity and Western blotting assays. Increased levels of p27, the putative ubiquitinated form of p27 (70 kDa; refs. 18, 22) and a cleaved PARP fragment (50 kDa). *C,* effects of Celastrol at the end point (*left*). Tumors were collected after 16 days of treatment, and the prepared tissue extracts were used for Western blotting assay. Increased levels of putative ubiquitinated form of Bax (47 kDa; ref. 32). *C,* C4-2B tumors treated with Celastrol at 3.0 mg/kg/d for 3 days, followed by measurement of TUNEL (see Fig. 6*D*) and AR (*right*). Levels of AR protein and a related p50 fragment. *D*, inhibition of PC-3 tumor growth by Celastrol (the second PC-3 experiment). Male nude mice bearing PC-3 tumors treated with 1.5 mg/kg/d Celastrol or Oridonin for 31 days. *Points,* mean tumor volume in each experimental group containing four mice; *bars,* SE; *, $P < 0.01$. *Arrow,* one tumor became undetectable after 5 days of Celastrol treatment.

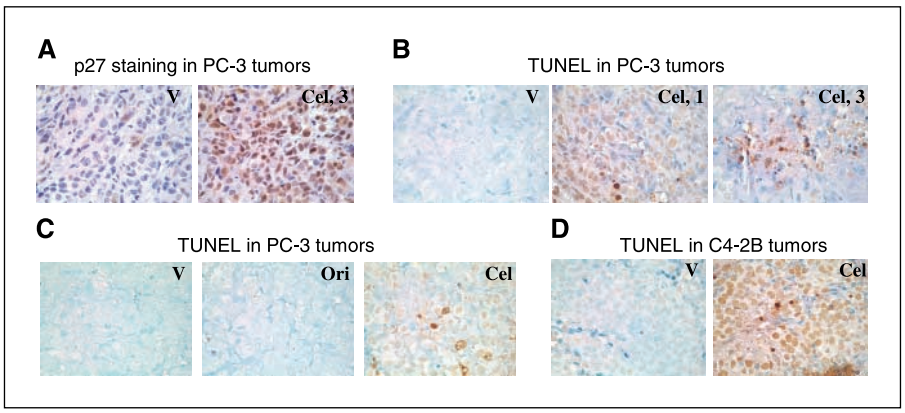

Figure 6. Immunohistochemistry and TUNEL assay using mouse tumor samples. *A* and *B,* p27 accumulation and TUNEL positivity in PC-3 tumors by Celastrol treatment (from the first PC-3 experiment; see Fig. 5*A*). Tumors were collected after treatment for 16 days, and the prepared tissue slides were used for immunostaining with p27 antibody (*A*) and TUNEL assay (*B*). *C,* TUNEL positivity in PC-3 tumors induced by Celastrol (from the second PC-3 experiment; see Fig. 5*D*). The slides prepared from the tumors treated with the vehicle (*V*), Oridonin (*Ori*), or Celastrol (*Cel*) were used for TUNEL assay. *D,* TUNEL positivity in C4-2B tumors induced by Celastrol. C4-2B tumors treated with Celastrol at 3.0 mg/kg/d for 3 days, followed by measurement of TUNEL positivity and AR (see Fig. 5*C*, *right*). Original magnification, ×400.

(33). Our previous studies have also indicated that the ester bond carbon of green tea polyphenol (−)-epigallocatechin-3-gallate or (−)-EGCG, its analogues, and tannic acid are essential for inhibition of the proteasomal chymotrypsin-like activity (24, 25, 34). In addition, our recent results suggested that a ketone carbon in dietary flavonoids is responsible for interacting in and inhibiting the proteasome (26). The current study supports the conclusion that conjugated ketone carbons C_2 and C_6 of Celastrol might contribute to its proteasome-inhibitory potency (Fig. 1). Celastrol potently inhibited the chymotrypsin activity of a purified 20S proteasome with an IC_{50} value of 2.5 μmol/L (Fig. 1*C*). In contrast, Oridonin, which has a different structure from Celastrol (Fig. 1*A*), did not inhibit the purified proteasome activity (Fig. 1*C*). Furthermore, Celastrol-mediated inhibition of the chymotrypsin-like activity seems preferable because it did not inhibit the proteasomal trypsin-like activity and was much less potent for PGPH-like activity under the same conditions (Fig. 1*D*).

When comparing the *in vitro* and *in vivo* potencies of Celastrol, we noted that 2.5 μmol/L Celastrol was needed to inhibit 50% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 1*C*), and the same concentration of Celastrol was needed for 40-55% inhibition of the chymotrypsin-like activity in intact prostate cancer cells (Figs. 2*A* and 4*A*). These results suggest that the proteasome is a relatively specific target for Celastrol in human prostate cancer cells.

The accumulation of IκBα, Bax, and p27 proteins in prostate cancer cells (Fig. 3) was due to the inhibition of the proteasome activity by Celastrol, which is supported by the following evidence: (*a*) as discussed above, Celastrol is a relatively specific, potent, proteasomal chymotrypsin inhibitor *in vitro* (Fig. 1); (*b*) Celastrol inhibits the proteasomal activity in cultured prostate tumor cells in both dose- and time-dependent fashions (Figs. 2-4); and (*c*) accumulation of IκBα, Bax, and p27 proteins was also observed in both concentration- and time-dependent manners (Fig. 3; data not shown). The following arguments support the idea that Celastrol-accumulated IκBα, Bax, and/or p27 proteins are proapoptotic (23, 35, 36) in the treated prostate cancer cells and tumors. First, when PC-3 cells were treated with Celastrol, the accumulation of IκBα, p27, and Bax proteins was increased either prior to or at the same time as apoptosis induction (Fig. 3). Second, accumulation of either unmodified or ubiquitinated forms of p27 or Bax were also found in Celastrol-treated tumors undergoing apoptosis (Figs. 5 and 6).

In the current study, Oridonin at up to 5 μmol/L, was unable to inhibit the proteasome activity and induce prostate cancer cell apoptosis (Figs. 2-4). This was consistent with the report from Leung et al., that Oridonin and other diterpenoids do not block the degradation of IκBα (37). Instead, Oridonin at up to 50 μmol/L, could directly interfere with the DNA-binding activity of nuclear factor κB to its response DNA sequence and also affect the translocation of nuclear factor κB from the cytoplasm to the nuclei (37). Furthermore, it has been reported that the ED_{50} values of Oridonin to inhibit the proliferation of PC-3 and LNCaP cells were 12 and 20 μmol/L, respectively (38). Apoptosis in LNCaP cells was only induced when Oridonin was used at 14 to 21 μmol/L (38).

Our data also show that i.p. treatment of PC-3 tumor–bearing nude mice with Celastrol resulted in the inhibition of proteasomal chymotrypsin activity in these tumors, accumulation of proteasome target proteins (i.e., p27), and induction of apoptosis (Figs. 5 and 6). Associated with this, significant inhibition (up to 93%) of tumor growth was observed (Fig. 5), possibly suggesting that the effective plasma levels of Celastrol have been reached. Impressively, one of the mice became, and remained, tumor-free to the end of the experiment after 1.5 mg/kg/d Celastrol treatment (Fig. 5*D*), suggesting that the long-term effect of the compound on tumors could be tumor regression. Furthermore, during the 31-day period of Celastrol treatment at 1.5 mg/kg/d, no overall gross toxicity was observed. Indeed, animals treated with Celastrol under these conditions showed no weight loss, decreased activity, or anorexia. More detailed microscopic and macroscopic pathologic studies are required to further document the lack of toxicity of Celastrol at these concentrations and under other different conditions. The fact that Celastrol targets the tumor cellular proteasome and could inhibit human tumor growth *in vivo* gives strong support for proof-of-concept of using proteasome inhibitors as novel anticancer drugs (16, 17). The remaining challenge is to further use Celastrol, or design, synthesize, and evaluate more potent and selective Celastrol analogues with little or no toxicity as proteasome inhibitors, to suppress human tumor growth in clinical settings.

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References

1. Fujiki H, Suganuma M, Kurusu M, et al. New TNF-α releasing inhibitors as cancer preventive agents from traditional herbal medicine and combination cancer prevention study with EGCG and sulindac or tamoxifen. Mutat Res 2003;523–4:119–25.
2. Cohen I, Tagliaferri M, Tripathy D. Traditional Chinese medicine in the treatment of breast cancer. Semin Oncol 2002;29:563–74.
3. Ahmed S, Anuntiyo J, Malemud CJ, Haqqi TM. Biological basis for the use of botanicals in osteoarthritis and rheumatoid arthritis: a review. Evid Based Complement Alternat Med 2005;2:301–8.
4. Setty AR, Sigal LH. Herbal medications commonly used in the practice of rheumatology: mechanisms of action, efficacy, and side effects. Semin Arthritis Rheum 2005;34:773–84.
5. Tao X, Younger J, Fan FZ, Wang B, Lipsky PE. Benefit of an extract of *Tripterygium wilfordii* Hook F in patients with rheumatoid arthritis: a double-blind, placebo-controlled study. Arthritis Rheum 2002;46:735–43.
6. Li H, Jia YF, Pan Y, Pan DJ, Li D, Zhang LX. Effect of tripterine on collagen-induced arthritis in rats. Zhongguo Yao Li Xue Bao 1997;18:270–3.
7. Li H, Zhang YY, Huang XY, Sun YN, Jia YF, Li D. Beneficial effect of tripterine on systemic lupus erythematosus induced by active chromatin in BALB/c mice. Eur J Pharmacol 2005;512:231–7.
8. Xu X, Wu Z, Xu C, Ren Y, Ge Y. Observation on serum anti-double stranded DNA antibodies of tripterine in systemic lupus erythematosus of (NZBxW)F1 mice. Ann Rheum Dis 2003;62:377–8.
9. Liu RL, Liu ZL, Li Q, et al. The experimental study on the inhibitory effect of tripterine on airway inflammation in asthmatic mice. Zhonghua Jie He He Hu Xi Za Zhi 2004;27:165–8.
10. Pinna GF, Fiorucci M, Reimund JM, Taquet N, Arondel Y, Muller CD. Celastrol inhibits pro-inflammatory cytokine secretion in Crohn's disease biopsies. Biochem Biophys Res Commun 2004;322:778–86.
11. Cleren C, Calingasan NY, Chen J, Beal MF. Celastrol protects against MPTP- and 3-nitropropionic acid-induced neurotoxicity. J Neurochem 2005;94:995–1004.
12. Allison AC, Cacabelos R, Lombardi VR, Alvarez XA, Vigo C. Celastrol, a potent antioxidant and anti-inflammatory drug, as a possible treatment for Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 2001;25:1341–57.
13. Chang FR, Hayashi K, Chen IH, et al. Antitumor agents. 228. Five new agarofurans, Reissantins A-E, and cytotoxic principles from *Reissantia buchananii*. J Nat Prod 2003;66:1416–20.
14. Zhou YX, Huang YL, Xu QN, Ye M, Sun CF, Zhou ZD. Several monomes from *Tripterygium wilfordii* inhibit proliferation of glioma cells *in vitro*. Ai Zheng 2002;21:1106–8.
15. Nagase M, Oto J, Sugiyama S, Yube K, Takaishi Y, Sakato N. Apoptosis induction in HL-60 cells and inhibition of topoisomerase II by triterpene celastrol. Biosci Biotechnol Biochem 2003;67:1883–7.
16. Goldberg AL. Functions of the proteasome: the lysis at the end of the tunnel. Science 1995;268:522–3.
17. Dou QP, Li B. Proteasome inhibitors as potential novel anticancer agents. Drug Resist Updat 1999;2:215–23.
18. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–5.
19. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature 1991;349:132–8.
20. Hiller MM, Finger A, Schweiger M, Wolf DH. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science 1996;273:1725–8.
21. Chen ZJ, Parent L, Maniatis T. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. Cell 1996;84:853–62.
22. An B, Goldfarb RH, Siman R, Dou QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 1998;5:1062–75.
23. Lopes UG, Erhardt P, Yao R, Cooper GM. p53-dependent induction of apoptosis by proteasome inhibitors. J Biol Chem 1997;272:12893–6.
24. Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*. J Biol Chem 2001;276:13322–30.
25. Nam S, Smith DM, Dou QP. Tannic acid potently inhibits tumor cell proteasome activity, increases p27 and Bax expression, and induces G1 arrest and apoptosis. Cancer Epidemiol Biomarkers Prev 2001;10:1083–8.
26. Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR, Dou QP. Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol 2005;69:1421–32.
27. Sun J, Nam S, Lee CS, et al. CEP1612, a dipeptidyl proteasome inhibitor, induces p21WAF1 and p27KIP1 expression and apoptosis and inhibits the growth of the human lung adenocarcinoma A-549 in nude mice. Cancer Res 2001;61:1280–4.
28. Marks LS, DiPaola RS, Nelson P, et al. PC-SPES: herbal formulation for prostate cancer [discussion 376–7]. Urology 2002;60:369–75.
29. Dorkin TJ, Neal DE. Basic science aspects of prostate cancer. Semin Cancer Biol 1997;8:21–7.
30. Arnold JT, Isaacs JT. Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. Endocr Relat Cancer 2002;9:61–73.
31. Lin HK, Altuwaijri S, Lin WJ, Kan PY, Collins LL, Chang C. Proteasome activity is required for androgen receptor transcriptional activity via regulation of androgen receptor nuclear translocation and interaction with coregulators in prostate cancer cells. J Biol Chem 2002;277:36570–6.
32. Li B, Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. Proc Natl Acad Sci U S A 2000;97:3850–5.
33. Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 1995;268:726–31.
34. Smith DM, Wang Z, Kazi ALLH, Chan TH, Dou QP. Synthetic analogs of green tea polyphenols as proteasome inhibitors. Mol Med 2002;8:382–92.
35. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–6.
36. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
37. Leung CH, Grill SP, Lam W, Han QB, Sun HD, Cheng YC. Novel mechanism of inhibition of nuclear factor-κB DNA-binding activity by diterpenoids isolated from *Isodon rubescens*. Mol Pharmacol 2005;68:286–97.
38. Ikezoe T, Chen SS, Tong XJ, Heber D, Taguchi H, Koeffler HP. Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. Int J Oncol 2003;23:1187–93.